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AD_____

CONTRACT NUMBER: DAMD17-94-C-4042

TITLE: Identification of Protective Brucella Antigens and Their Expressions in Vaccinia Virus to Prevent Disease in Animals and Humans

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REPORT DATE: May 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1996	Annual (1 May 95 - 30 Apr 96)

4. TITLE AND SUBTITLE
Identification of Protective Brucella Antigens and Their Expressions in Vaccinia Virus to Prevent Disease in Animals and Humans

5. FUNDING NUMBERS
DAMD17-94-C-4042

6. AUTHOR(S)
Gerhardt G. Schurig, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Virginia Polytechnic Institute
Blacksburg, Virginia 24061-0249

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, May 96). Other requests for this document shall be referred to Commander, U.S. Army Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Vaccinia-*Brucella* recombinants expressing the *Brucella* GroEL, HtrA and Cu/Zn SOD proteins were used to immunize mice and the animals were tested for protection against a virulent *B. abortus* challenge. The Wyeth vaccinia strain expressing GroEL recombinant was unable to proliferate in mice and did not protect. The Western Reserve vaccinia strain expressing HtrA or SOD were able to replicate in mice but did not protect after 1 or 2 vaccinations. It was not possible to detect a CMI response to HtrA or SOD in these mice which may account for lack of protection. A synthetic early-late promoter is now being used to generate vaccinia constructs in order to overcome this problem. Homologs of the *E. coli* YajC and SecD were identified in *Brucella* and vaccinia recombinants are being generated using the corresponding genes. Genes for the *Brucella* 18kDa lipoprotein and a 14kDa protein have been cloned to create vaccinia recombinants with them. The Baculovirus expression system is being employed to produce large quantities of *Brucella* antigens for *in vitro* and *in vivo* testing. Fractionation of *Brucella* lysates is being used as an alternative method to find protective antigens; 4 additional antigens have been identified by this method.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Brucella, vaccinia recombinant, protection, antigens, immunity, vaccine	30
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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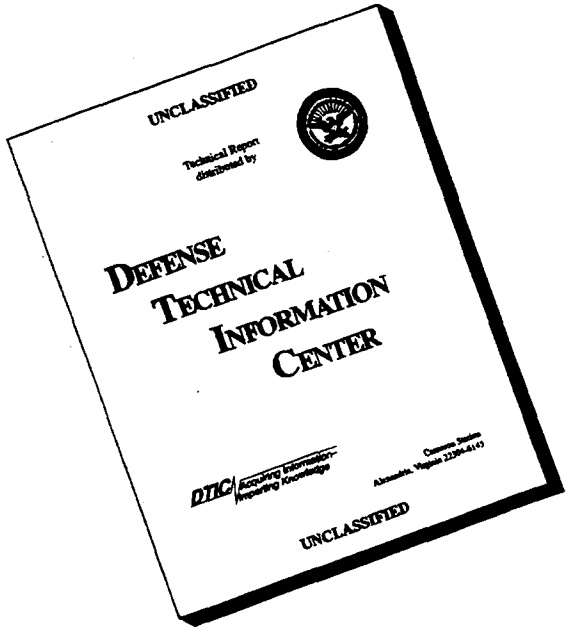

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05/25/96

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III. Table of Content.

V. INTRODUCTION.

a. Nature of the problem, background and previous work.

Brucellosis affects millions of people worldwide. Humans contract the disease either by consuming infected foods or by coming in contact with animals shedding the organism. The disease is characterized by an undulant fever, cold sweats and general malaise; any exercise will produce pronounced fatigue. If untreated the disease can last from a few weeks to several years. Serious complications leading to death can occur. Tetracycline is the treatment of choice for infected humans. In severe cases tetracycline treatment is supplemented with streptomycin or rifampicin. Brucellosis affects a variety of animals including swine, cattle, sheep, goats, dogs, and camels. Important species of *Brucella* are: *suis, abortus, ovis, melitensis, canis*, and *neotomae*, each with certain predilection for a particular animal species.

Humans are susceptible to *B. melitensis, B. suis, B. abortus* and *B. canis* in decreasing order. The disease is endemic in many Middle East countries, Asia, Mexico, Central and parts of South America.

Antibiotic resistant mutants of *Brucella*, including tetracycline, rifampicin and streptomycin resistant ones, are not difficult to produce in the laboratory. Such mutants could be utilized in biological warfare resulting in high morbidity with no adequate treatment. Therefore, prevention of infection through vaccination is desirable.

Currently available vaccines can not be used in humans because of their side effects or lack of effectiveness. The current, most used vaccine for protecting cattle against *B. abortus* is strain 19 and for protecting goats against *B. melitensis* is strain Rev 1. Vaccination with these strains leads to seroconversion complicating diagnosis of the disease. Both of these vaccine strains are pathogenic for human beings. *B. abortus* strain RB5I was developed in our laboratory and is now replacing strain 19 as the vaccine of choice for bovine brucellosis. This strain is a stable, rough natural mutant devoid of 0-side chain. The strain is able to induce protective immunity in mice (1) and cattle (2,3) without the induction of antibodies to the 0-side chain (1,2). Transfer of T cells from strain RB5I immunized mice will transfer protective immunity against a virulent challenge (4), similar to the immunity transferred to mice by T cells derived from strain 19 immunized mice (5). This confirms previous indications that immunity to *B. abortus* infection requires a strong CMI component (4,5,6,7,8). The specific antigens which confer strong protective CMI have yet to be defined.

Macrophages are the principle cells of residence for *Brucella* in infected animals. Resistance usually depends on the correct interaction between T lymphocytes, specific for particular bacterial antigens, and the macrophage (9,10,11,12,13,14). Activation of

macrophages by interferon-gamma (INF-g) will lead to the destruction of intracellular *Brucella* (4). This further suggests that T cells, particularly those responsible for the production of INF-g (T helper1-CD4+ response) are of major importance in anti-*Brucella* immunity (4). These observations do not eliminate a concomitant protective role for cytotoxic T cells (Tc, CD8+), since protective immunity can be demonstrated by passive transfer of either Th or Tc cells (8). It is possible that in Brucellosis the major role of the Tc cells in protection is production of INF-g and not direct cytotoxicity. Considering these observations, it is most likely that *Brucella* proteins involved in protective immunity will preferentially stimulate INF-g producing T cells which in turn activate macrophages enhancing their brucellacidal capabilities. Therefore, if *Brucella* proteins with such characteristics could be identified, they would be most likely to induce a strong and protective CMI response. They could be used in cloning and expression systems that are able to present these antigens appropriately to the immune system.

b. Purpose of current work.

This project intends to identify *Brucella* antigens which are likely to stimulateTh1 responses (with production of INF-g) with lymphocytes from vaccinated mice. Once such antigens have been identified, the genes coding for such antigens will be isolated and vaccinia recombinants created. These recombinants will be used to immunize mice to determine whether they can induce a humoral and/or CMI response and protect mice against a challenge with virulent *B. abortus*.

c. Outline of approach

The methodology used can be outlined as follows (see summary flow chart in appendix):

i). Express *Brucella* antigens in genomic expression libraries and detect potentially protective antigens by either or both:
   1. The use of specific antibodies of the IgG2a subisotype found in immunized animals (IgG2a is considered as a potential indicator of Thl involvement) and
   2. The use of lymphocytes from animals immunized with the protective *B. abortus* strain RB5l vaccine. Lymphocytes will be tested for their ability to proliferate and produce INF-γ upon exposure to such antigens.

ii). Fractionate *Brucella* extracts and detect antigens which stimulate lymphocytes from vaccinated mice to produce INF-g. The vast majority of such antigens are expected to be proteins. Partial amino acid sequence of the amino terminal end of these proteins will be determined.
Complimentary oligonucleotide probes will be synthesized and used for the identification of the gene using Southern blots. Alternatively, antibodies will be

raised against the selected antigens and gene libraries will be screened by colony and western blot techniques to identify genes responsible for the expression of the antigens.

iii). Subclone such genes identified by the above outlined procedures into vaccinia virus (Western Reserve and/or Whyet strains). Test the recombinants for their ability to express the antigens ***in vitro*** and their ability to induce a protective immune response in mice. Analyze the character of the immune response induced by the recombinant vaccine and if protective, analyze in detail the immunological parameters associated with protection.

VI. BODY.

1. Preparation of reagents.

a. Antisera.

During the first year of the project, 2 proteins (10.2 kDa and 11.6 kDa) present in an ammonium sulfate extract of strain RB51(fraction S2) were identified which stimulated production of INF-g by proliferating lymphocytes from RB51 vaccinated mice. The N-terminal amino acid sequences were determined. Amino acid sequences were back translated using the preferred codon usage of *Brucella* and oligonucleotides probes generated were digoxigenin labeled. The oligonucleotide sequences used for the generation of probes were:

i.) for the 10.2kDa amino acid sequence :

gccttcgcctaagtggtgggttaccgccatggcctggaagaaggtt

ii) for the 11.6 kDa amino acid sequence:

gcccagccgatgttcatcgttctgctgtagacgttgttggcgtt

Unfortunately both the oligonucleotides probes failed to hybridize to the *Brucella* chromosomal DNA. As an alternative method we are screening our *Brucella* genomic library for the presence of these proteins. We have prepared a specific antisera to these 2 proteins in goat (goat 52) since mice vaccinated with strain RB51 do not produce antibodies to these 2 proteins, although they do develop a good *in vitro* lymphocyte response to them. Goat (52) was immunized with the two specific proteins obtained by cutting the corresponding bands out of SDS-PAGE preparations of strain RB51 fraction S2. The bands were minced in physiological saline and emulsified in Freund's incomplete adjuvant. The goat was immunized subcutaneously 3 times with this preparation. The resulting antisera reacted very strongly with the 10.2 and 11.6 kDa proteins. This antiserum, absorbed with the appropriate *E. coli*, is being used to screen our existing genomic libraries.

b. Antigen purifications.

In order to test *in vitro* lymphocyte reactivities or *in vivo* delayed hypersensitivities (DH) against specific *Brucella* antigens, it is important to have significant amounts of

purified antigens. Our experience up to this point indicates that this is not an easy task.The purified antigens are mainly needed to clearly define the immune responses in our recombinant vaccinia immunized mice. They could also be used to decide if a selected antigen is indeed eliciting the appropriate responses *in vitro* with lymphocytes from *Brucella* vaccinated mice. Purified antigens can be obtained directly from *Brucella* by appropriate fractionation methods, from recombinant *E.coli* expressing the antigen, as fusion proteins or from recombinant Bavulovirus (BV; see last part of this section). We are preparing some of the antigens as fusion proteins and have initiated cloning into BV. The BV cloning and expression system should enable us to get large amounts of the specific *Brucella* antigens. At present our most reliable (although not perfect) system to obtain reasonable amounts of selected antigens is through fractionation of *Brucella* strain RB51 or *E.coli* recombinants expressing the appropriate*Brucella* antigen. Briefly, the method developed is as follows: lysates of *Brucella* or recombinant *E.coli* are separated by SDS-PAGE, the appropriate band is cut out and electroeluted. The electroeluted antigen is mixed with Biorad Beads, centrifuged and the supernatant is saved (this removes residual SDS). The supernatant is thereafter mixed with polymyxinB beads, centrifuged and the supernatant removed (this removes any residual LPS) and lyophilized. The lyophilized antigen is dissolved in appropriate buffers for use in the different assays. Before use, protein concentration is determined. The following antigens have been and are being prepared by this method:

a. Using recombinant *E. coli* : GroEL, HtrA, CuZn SOD, 18kDa antigen and 14 kDa antigen

b. Using strain RB51 overexpressing the SOD(RB51 with pBBSOD): CuZn SOD .

Proteins YajC and SecD will be obtained as fusion proteins (see.2. selection of antigens section).

Using eukaryotic as opposed to prokaryotic cloning and expression systems can be advantageous in obtaining large amounts of *B. abortus* proteins. Baculovirus (BV) as a cloning and expression system has the following advantages: non-essential gene products polyhedrin and p10 proteins, very strong promoters for polyhedrin and p10 driving the synthesis of large quantities of proteins, temporal expression factors that decrease the toxicity of the replicating BV recombinants, inserts accepted up to 200 Kbp, and faithful post-translational processing. The highest expression levels reported using baculovirus expression vector is 25 - 50% of the total cellular protein, corresponding to approximately 1 gram of protein product per 10^9 cells.

While vaccinia virus is an excellent cloning and expression system for *in vivo* immunization of various species, vaccinia virus recombinants do not produce large quantities of proteins *in vitro.* In contrast, the baculovirus expression system generates pure recombinant proteins *in vitro* in sufficient quantities for analysis of their basic characteristics and for use in *in vivo* and *in vitro* immune responses.

Using the BAC-TO-BAC TM Baculovirus Expression System (GibcoBRL, Grand Island,

NY) recombinant bacmids (baculovirus DNA propagated in E.coli as a plasmid) were generated by subcloning *B. abortus* genes into the pFASTBAC1 and introducing the plasmids into competent *E.coli* (where they are transposed into the bacmid resulting in recombinant bacmids). Successful cloning was demonstrated by isolation, restriction enzyme (RE) digestion of the recombinant bacmids, separation of the RE fragments by agarose gel electrophoresis and hybridization of digoxygenin-labeled probes to the transfered RE fragments.
Five *B. abortus*- pFASTBAC donor plasmids were generated for the following *B. abortus* proteins: GroEL, GroES, GroES-EL (partial GroEL-complete GroES and complete GroEL-complete GroES) and HtrA . Constructs are depicted on figures in appendix.
The pFASTBAC recombinant donor plasmids were transfected into competent E. coli DH10BAC cells carrying the baculovirus bacmid. Recombinant bacmid DNA was isolated from white colonies and purified. The presence of the bacmid DNA in undigested bacmid mini-preps was indicated by the appearance of a band that migrated slower in 0.5 % agarose gel than the 23.1 kb fragment of the appropriate marker. The identity of the cloned *B. abortus* genes was confirmed by Southern analysis using digoxygenin -labeled *groES-groEl* and htrA DNA to blotted RE digests of the pFASTBAC donor plasmids and recombinant bacmids.

2. Selection of antigens.

The selection of GroEL, HtrA and CuZn SOD as *Brucella* antigens to be cloned into vaccinia for protection studies was reported in the previous report. Several reasons indicate a potential role for these antigens in the protective immune response.GroEL is able to induce IgG2a antibodies, lymphocyte proliferation and INF-g production in *Brucella* exposed mice (Table 1); HtrA is able to induce IgG2a antibodies (Table 1) and CuZn RB51deletion mutants are as attenuated as the parent strain but have significantly reduced protective abilities suggesting a role of SOD in protection (unpublished, confidential information). Also, delayed hypersensitivity tests using SOD as the test antigen in RB51 vaccinated mice suggest induction of a Th1 response. In addition, no antibodies against SOD are found in vaccinated animals.

We also reported previously the identification of 15 clones in our library which were expressing *Brucella* antigens identified by antibodies from vaccinated mice and which appeared to be active in the LTA and were able to induce INF-g production by lymphocytes from vaccinated mice. Clones positive with mouse sera demonstrated reactivity with anti-mouse IgG2a suggesting that the expressed antigens may play a role in a Th1 response.

During this year, further screening of lysates of the clones in both LTA and INF-g (ELISA based) assays still did not yield clear and conclusive results due to high background levels probably induced by *E. coli* LPS and LPS associated proteins, as adsorption of lysates with polymyxin B could not remove all of the problem. All clones screened in these assays were constructed using plasmid pBBR1MCS. The genes

were subcloned into the higher copy number plasmid pBluescript to increase expression of the putative antigens and in doing so, facilitate detection in LTA and INF-γ assays, however the background problem was not overcome. These results again stress the necessity of working with highly purified antigens to clearly determinate if a CMI is present.

The 15 clones were restriction enzyme mapped and this revealed 3 groups of clones:
group 1: Containing a 2.7 kb fragment
group 2: Containing a 2.9 kb fragment
group 3: Containing a 3.8 kb fragment consisting of a 1.1 and a 2.7 kb fragments when digested with the enzyme originally used for cloning (ClaI)

Southern blot analysis indicated that the 2.7 kb fragment from groups 1 and 3 clones were the same.

One of the clones from group 1 (clone MCB68) was selected for further study. The 2.7 kb fragment was sequentially made smaller (deletion mutants) and the whole 2.7 kb insert was sequenced. Sequence analysis indicates that the identity of the insert is part of the SecD operon. The genes encoded are thought to be *yajC* and *secD* based on sequence analysis (see figure appendix; *B. abortus* sequence in similarity arrangement with *secD* of *E.coli*, *H. influenzae*, *M. leprae* and *S. coelicolor*). Highly related organisms have a high degree of *secD* and *yajC* sequence similarity (*E. coli* and *H. influenzae* approx. 60% similarity) whereas more unrelated organisms have low similarity (*E. coli* and *M. leprae* approx 13.5% similarity). The *Brucella* and *E. coli* sequences were 25% similar (see figures appendix). In *E. coli* ,*secD* encodes for a 67 kDa SecD protein responsible for membrane transport; *yajC* encodes a hypothetical protein of 12 kDa of unknown function.

Primers homologous with the 5' and 3' ends of both *secD* (set1) and *yajC*(set2) were made and amplification of each gene was done using polymerase chain reaction (PCR). In order to express the respective proteins , *yajC* and *secD* were subcloned into the fusion protein expression vector pRSET A, B and C in order to have all 3 reading frames present. Using these vectors, a protein fused to a polyhistidine should be made, allowing affinity purification of the product. This system did not yield a product which may be due to an error in primer design. New primers for both *yajC* and *secD* were made; this time cloning into pmal-p2 or pmal-c2 is being done to obtain a maltose fusion protein (vector used depends on where the protein produced ends up in *E. coli*-cytoplasmic or membrane location). Once expression of *yajC* and *secD* is obtained, the affinity purified proteins will be tested for CMI reactivity with lymphocyte from RB51 vaccinated and unvaccinated mice.

After growth on minimal media, the morphological appearance of the clones derived from the 3 groups is different. The lack or addition of the 1.1 kb fragment to group 1 or group 3 clones alters colony morphology.The 1.1 kb fragment has been subcloned

into pBluescript and sequence analysis is being done.
Deletion mutants of the 2.9 kb fragment are being made; the 2.9 kb insert will then be sequenced. SecD and yajC have been cloned into vaccinia vector pMCO2 (recently obtained from Dr. Moss' laboratory at NIH) which has a synthetic early-late promoter . These constructs are currently being transfected into vaccinia.

We have also selected as candidates for subcloning into vaccinia, genes that encode a 18 kDa lipoprotein from the *Brucella* cell wall and a 14 kDa protein that encodes the C-terminal section of a 60kDa membrane protein. The major reason for selecting the 18 kDa protein is that it elicits a strong IgG2a response in RB51 vaccinated mice and IgG responses in a variety of animal species infected with *Brucella spp.* It is also able to induce the *in vitro* production of INF-γ with lymphocytes of RB51 vaccinated mice (Table 1).

The 14 kDa protein is produced by a clone originally identified from our Cla *B. abortus* genomic library (clone pBAI4). This has been subcloned into pRSet creating the clone pRS44.8 (courtesy of Dr. Marty Roop, Louisiana State University, LA). It contains the 1.8 kb Brucella DNA fragment encoding the C-terminus portion of the *Brucella* protein. Western blot analysis of *B. abortus* and *B. melitensis* was performed using antibodies raised against the fusion protein. The results showed that the antibodies recognized a native *Brucella* protein of approximately 60 kDa. Therefore, the fusion protein is thought to encode for a larger outer membrane protein. The fusion protein (BA-14kDa) from the original construct, pBAI4, was shown to be reactive with mouse and goat IgG (Table 1). It was also shown to be reactive with IgG of naturally *Brucella* infected cattle, dogs, and humans. The recombinant 14 kDa *Brucella* protein has been shown to be immuno-reactive both with antibodies and T-lymphocytes from experimentally and naturally infected animals (Table 1). Specific antibodies raised against this fusion protein was found to react with cell extracts from *B. melitensis* 16M, *B. abortus* 2308, and *B. abortus* S19.

Blast search using the DNA Star program yielded no significant matching sequences in all six reading frames. Primer selection was performed manually and checked using DNAStar primer selection program. Both forward and reverse primers were designed to contain a Sal I site. The forward primer is located - 19 from the first *Brucella* codon. The 23 oligomer contains a total of 7 codons. The sixth codon was changed from (ATC) to Met (ATG) to accommodate the need for a start codon in frame for the 14 kDa protein. This was essential for subcloning this insert into pMCO2. This vector with T7 promoter needs the start codon for expression of foreign proteins. The reverse primer with a *Sal* I site is a 24-mer oligo selected using site approximately 57 bases downstream 3' to the stop codon. Both the forward and reverse primers were analyzed for dimer formation and hairpin formation at 25C. Neither showed significant dimer formation (<2bp) or hairpin formation. The PCR product is approximately 385 bp as predicted from the sequence information. The PCR fragment will now be subcloned into pMCO2 using Sal I sites both on the primers and the vector. The ligated mixture will be used to transform E. coli. Clones expressing the 14 kDa protein will be selected

using colony blot assays and confirmed by Western blot analysis with appropriate controls. The plasmid pMCO2 will then be used to generate recombinant Vaccinia expressing the 14 kDa protein by transfection. Recombinant Vaccinia will be used to immunize mice for protection experiments.

Table 1. Immunological reactions detected with selected Brucella antigens using sera or lymphocytes from *Brucella* exposed mice.These antigens have been or are currently being cloned into vaccinia.

Immune responses to selected *Brucella* antigens after immunization of BALB/c mice with vaccine strain RB51

	Specific IgG2a found in sera	Induces lymphocyte proliferation *in vitro*	Induces production of INF-γ *in vitro*
YajC	+	+ (S)	+(S)
SecD	+	+ (S)	+(S)
18kDa	+	-	+
14kDa	+	+	+
GroEl	+	+	+

S = Suspect (assays positive in preliminary tests but need definitive confirmation with purified antigens)

Fractionation of *B. abortus* strain RB5l as an additional method to detect immunoreactive antigens.

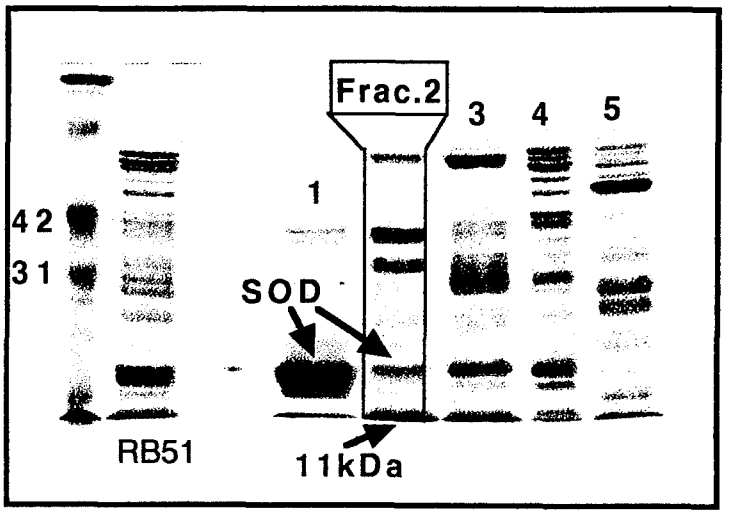
Proliferation studies using lysates from live and killed *B. abortus* strai RB51, showed that they were able to stimulate splenic lymphocytes from strain RB5 1 immunized mice to proliferate and produce INF-g. These studies also showed that cell lysates from killed RB51 were relatively more stimulatory than cell lysates from live RB51. Therefore, radiation killed RB51obtained from the National Animal Disease Center (NADC) were used. These cells were lysed in a l0 mM phosphate buffered saline pH 8.0, containing three protease inhibitors (PBS-3Pl). Cell lysis was accomplished by sonication using five cycles of 3 minutes on and 3 minutes off on ice at 70% power with a microtip (Heat Systems Inc). The cell lysate was clarified by centrifugation at l2,000Xg for 30 minutes and then filter sterilized using a 0.22uM membrane. The

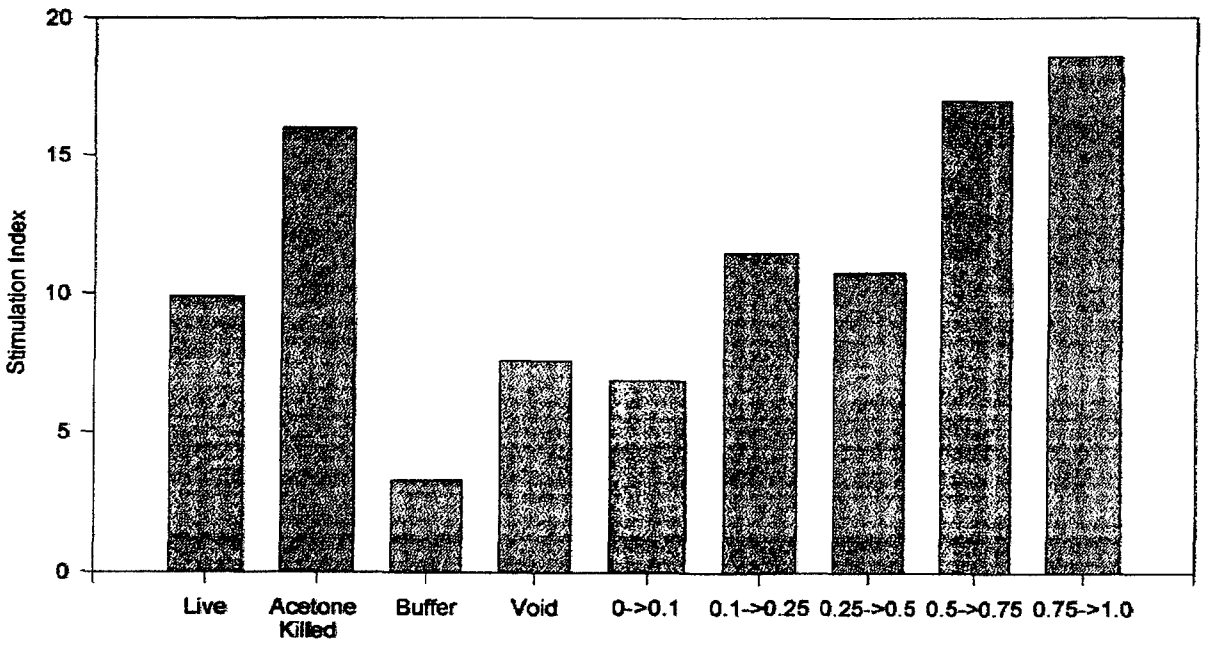
lysate was separated by ultracentrifugation at 100,000 Xg for 3 hours into ribosomal and supernatant fractions. INF-γ production assays showed that the majority of the stimulatory effect was found in the clear supernatant rather than in the ribosomal fraction. Fractionation was performed using the clarified lysate. Initial studies using the monoQ column in an FPLC system indicated that proteins that were eluted in the 0.1 to O.25M sodium chloride range were stimulatory. This fraction also produced distinct bands of proteins in an SDS-PAGE. In order to increase the yield, the fractionation was performed on a Q-sepharose FF anion exchange column (Pharmacia Biotech) using a similar salt step gradient (0 to 0.IM; 0.1 to 0.25M; 0.25 to 0.5M; 0.5 to 0.75M, and 0.75 to I.0M). This was a preparative scale-up column for the monoQ FPLC analytical column reported earlier. These step fractions were dialyzed against 0. IX PBS-3PI and each fraction along with appropriate controls were tested for their ability to stimulate proliferation of splenic lymphocytes from RB51 immunized mice (Figure 1).

In order to determine the potential stimulatory effect of nucleic acids present in the fractions, each of the fractions was treated with DNase and RNase and tested simultaneously in the proliferation assay. Treatment of fractions with nucleases dramatically reduced the stimulatory activity. The results demonstrate that the nucleic acids found in the 0.25 to I.0M salt fractions were largely responsible for the stimulatory effects of these fractions, whereas the 0.0 to 0.25M fractions had lower levels of the nucleic acids, or nucleic acid present in these fractions were not primarily responsible for the stimulatory effect. SDS-PAGE analysis of these fractions are shown in figure1. From the results we can conclude that the 0.1 to 0.25 M fraction had the highest relative specific stimulatory activity, especially after the treatment of the fraction with nucleases. This fraction also had the most distinct protein banding pattern, and a total of five major bands were discernible, compared to 10 to 20 bands observed in the other fractions. One of the bands is CuZn SOD (figure1). When this fraction was analyzed on a native gel, it produced 4 major constituent protein bands. Therefore, further separation and characterization of the proteins in this fraction was under taken using preparative electrophoresis.

The 0.1 to 0.25 M salt fraction was dialyzed, concentrated, and electrophoresed on a 5.5 cm non-denaturing preparative acrylamide gel and 1 ml fractions were collected at the rate of 100 ul/min after elution of the tracking dye. A total of 69 fractions were collected by the end of the fractionation. One hundred microliter aliquots from each of the fractions were collected in to 7-one ml pools. They were dialyzed and concentrated by freeze drying. These seven pools are being analyzed by SDS-PAGE and will be tested for their ability to induce proliferation and INF-γ production using lymphocytes from RB51 vaccinated mice.

Figure 1. SDS-PAGE analysis of killed *B. abortus* strain RB51 fractions and their ability of fraction 2 (0.1-0.25M salt gradient) to induce lymphocyte proliferation with lymphocytes obtained from RB51 vaccinated mice.Fraction 2 (0.1-0.25M salt gradient) was selected for further purification.





Preparation of new vaccinia/ Brucella recombinants.

Recombinant WySBGroEL. Plasmid pBA2168BC was digested with *EcoR*I, *Sal* I and *Dra*I and overhanging ends were filled in using Sequenase. The resulting 1.7 kb band containing the *B. abortus groEL* gene was cloned into the *Sma*I site of pSC11 shuttle vector. This construct, pSBgroEL, was used to transform *E. coli* DH5α using the calcium chloride method and transformants were detected by DNA analysis. Plasmid pSBgroEL was reisolated from *E. coli* DH5α and used for transfection of HuTK$^-$ cells. HuTK$^-$ cells were seeded into a T25 flask (Corning) at 40,000 cells/cm^2. One ug of plasmid DNA was ethanol precipitated and allowed to dry under sterile conditions, then resuspended in 50 ul OPTI-MEM medium. HuTK$^-$cells were infected with the New York Board of Health small pox vaccine strain Wyeth, and incubated for two hours at 37 C with 5% CO_2. In a sterile polystyrene tube, 50 ul of undiluted Lipofectin reagent (Gibco) was mixed with the 50 ul plasmid DNA, and incubated at room temperature for 15 minutes. The entire 100 ul mixture was added to the cells after removal of the virus from the cells and addition of 3 ml OPTI-MEM. Following 16 hours of incubation, growth media was added, the cells were incubated and observed daily for cytopathic effect (CPE). At 4+ CPE, the flasks were freeze-thawed three times, followed by a brief sonication. The virus was purified by 3 consecutive plaquing in HuTK cells and the recombinant was termed WySBGroEL.

Analysis of protein expression and immunologic cross-reaction with GroEL homologous from other bacteria was carried out by Western blot analysis using the following primary antibodies: *B. abortus* specific hypeimirnune goat (No. 48) serum , a rabbit antiserum against *Legionella pneumophilia* HtpB protein, a rabbit antiserum against *Helicobacter pylori* CPN60 protein, a mouse monoclonal antibody against *E. coli* GroEl and a rabbit monospecilic antiserum against *E. coli* GroEL sera and the appropriate peroxidase conjugated species-specific secondary antibodies.

The western blot analysis using goat 48 serum detected a 60 kDa protein both in strain RB51 extract and recombinant WySBGroEL. All antibodies recognized the *E. coli* GroEl in the non-transfected DH5α *E. coli.* and in the pSBGroEL preparations. Each antibody, excepting the anti *E. coli* GroEL monoclonal antibody, also recognized the GroEL in the WySBGroEL recombinant and strain RB51 preparations. The non-transfected HuTK cells, the Wyeth vaccinia virus and the recombinant Wyth carrying only the plasmid pSC 11 were negative against all antibodies. The results indicated that: 1) the vaccinia virus recombinant expressed the *B. abortus* GroEL protein, 2) the *B. abortus* GroEL had cross-reactivity with homologos from other bacteria 3) the *E. coli* monoclonal antibody was directed against an epitope of the *E. coli* GroEL which did not exist on the *B. abortus* GroEl. The recombinant was tested in mice.

The following vaccinia recombinants (using WR strain of vaccinia and using mainly shuttle vector pMCO2) are in various stages of development :

GroEL recombinants. Testing of the humoral immune response against the WySBGroEL (see elsewhere) indicated that this recombinant was not an efficient immunogen in mice. This observation corresponds with those of other workers concerning recombinants developed with the Wyeth strain. Therefore the pSBgroEL plasmid is being subcloned into the Western Reserve (WR) strain.

GroES-EL recombinant. The genes of *B. abortus* tandem chaperones GroES-EL were cloned in the shuttle plasmid pN'U601. Development of this vaccinia virus recombinant is presently a low level priority.

YajC and secD recombinants. Genes of these proteins are being used to produce the respective vaccinia WR recombinants.

SOD recombinant. Cloning of the *B. abortus* SOD gene using shuttle vector pMCO2 is being carried out.

18kDa recombinant. The 18 kDa *B. abortus* lipoprotein gene is being used to produce the respective vaccinia WR / Brucella 18 kDa recombinant.

14 kDa recombinant. The 14 kDa *B. abortus* protein gene is being used to produce the respective vaccinia WR / Brucella 14 kDa recombinant.

Vaccination trials with vaccinia/Brucella recombinants.

The following vaccinia/ Brucella recombinants were used for protection studies: WySBGroEL (described above), pSBHtrA (vaccinia WR expressing *Brucella* HtrA - construction described last year) and pUBSOD (vaccinia WR expressing *Brucella* CuZn SOD - construction described last year). Construction of the plasmids used to transfect vaccinia to derive these recombinants are illustrated in the appendix.

BALB/c mice were vaccinated once with the vaccinia recombinant and challenged with virulent *B. abortus* strain 2308 seven weeks later or vaccinated twice 3 weeks apart and challenged four weeks after the second vaccination. Mice were killed at 1 week post challenge and colony forming units (CFU) of *Brucella* per spleen enumerated and expressed as $\log_{10}$ values. Animals were bled before vaccination and at various intervals thereafter. The dose of vaccination was 10^7 median tissue culture infective doses $(TCID)_{50}$ recombinant viruses in 0.1 ml PBS intraperitoneally.

WySBGroEL recombinant did not replicate in mice based on lack of antibody production against both, vaccinia proteins and the *Brucella* GroEL. This observation corresponds with those of other workers concerning recombinants developed with the Wyeth strain. The other 2 recombinants did replicate in mice as reported previously (Veterinary Microbiology 45:171-183, 1995) and produced antibodies to vaccinia proteins. The HtrA recombinants induced antibodies to *Brucella* HtrA but the SOD recombinant did not produce antibodies to the *Brucella* CuZn SOD. None of the

recombinants protected against challenge with virulent *Brucella* while vaccination with*B. abortus* vaccine strain RB51 induced significant protection (Table 2). The lack of protection by the GroEL recombinant was not unexpected since it did not replicate in mice and therefore, we are constructing recombinants using the vaccinia WR strain which replicates well. Two vaccinations with the recombinant expressing HtrA enhanced infection with the challenge strain suggesting that the HtrA antigen may have a deleterious effect on the protective immune response to *Brucella*. The data also indicate that two inoculations with vaccinia, 3 weeks apart will induce some level of non-specific immunity to *Brucella* (see statistically significant difference among groups 3, once vaccinated or twice vaccinated). Data also confirms previous observations that two immunizations with RB51 lead to higher protection levels than 1 vaccination.

Analysis of the immune response to *Brucella* antigens induced by the other 2 recombinant vaccines (HtrA and SOD) indicated a strong anti-HtrA antibody response but did not reveal anti SOD antibodies. The anti HtrA antibody response is not expected to be protective since passive transfer of anti HtrA antibodies does not confer protection. Potentially, a CMI response to either HtrA or SOD could be protective since T cell transfer experiment with RB51 sensitized T cells is protective in mice. Therefore, we tested recombinant vaccinated mice for a CMI response *in vitro* using lymphocyte transformation assays (LTA) and INF-γ production and *in vivo* using foot-pad DH reactions.

Mice vaccinated once with the HtrA and SOD vaccinia recombinants and the appropriate controls were tested at 7, 11 and 13 weeks post immunization using the corresponding purified antigens. We were unable to demonstrate a definitive *in vivo* or *in vitro* CMI response in any of the vaccinia vaccinated mice. These data suggest that the present constructs are not inducing CMI against either HtrA or SOD which may explain the lack of protection. We believe that the failure to induce a strong and detectable CMI response to the *Brucella* antigens by the vaccinia recombinants may be due to the type of promoter we have used in our constructs i.e they do not lead to high expression of the recombinant proteins. We are now proceeding to use a synthetic early-late promoter supplied by Dr. B. Moss' laboratory to greatly enhance the synthesis of the recombinant protein by vaccinia particularly during the early stages of the viral replication cycle.

Table 2 Protective effects after 1 or 2 immunizations with vaccinia recombinants and vaccine strain RB51.

1 vaccination

Group	Vaccination day 0	Challenge day 49	Serology at challenge antibodies to:	log spleen day 56
1	psbHtrA	2308	Vaccinia & HtrA	5.09*
2	psbGroEL	2308	negative	5.33*
3	psc11	2308	Vaccinia only	5.57*
4	pUBSOD	2308	Vaccinia only	5.44*
5	RB51	2308	various Brucella antigens	3.62

2 vaccinations

Group	vaccination day 0 & 21	challenge day 50	serology at challenge antibodies to:	log spleen day 57
1	psbHtrA	2308	Vaccinia & HtrA	5.77**
2	psbGroEL	2308	negative	4.96
3	psc11	2308	Vaccinia only	4.37
4	RB51	2308	various Brucella antigens	2.47

** statistically different from groups 3 and 4.

Group 3, 1 vaccination different from group 3, 2 vaccinations p = 0.0477

* Statistically not significant

Mice vaccinated once with the HtrA and SOD vaccinia recombinants and the appropriate controls were tested at 7, 11 and 13 weeks post immunization using the corresponding purified antigens. We were unable to demonstrate a definitive *in vivo* or *in vitro* CMI response within any of the recombinantvaccinia immunized mice. These data suggest that the present constructs are not inducing CMI against either HtrA or SOD which may explain the lack of protection. We believe that the failure to induce a strong and detectable CMI response to the *Brucella* antigens by the vaccinia recombinants may be due to the type of promoter we have used in our constructs i.e they do not lead to high expression of the recombinant proteins. We are now proceeding to use a synthetic early-late promoter supplied by Dr. B. Moss' laboratory to greatly enhance the synthesis of the recombinant protein by vaccinia particularly during the early stages of the viral replication cycle.

VII. CONCLUSIONS.

We have identified additional Brucella antigens which may have a role in protective immunity based on their *in vitro* activities with serum or T cells from strain RB51 vaccinated mice. The genes for these proteins have been identified and are available. The additional proteins are: YajC, SecD, 18kDa lipoprotein and 14 kDa protein. These proteins together with previously described proteins GroEL, HtrA and Cu/Zn SOD are being expressed or will be expressed by recombinant vaccinia using a synthetic early - late promoter which should lead to higher antigen production.

We have determined that the Wyeth strain of vaccinia is not a good vector at least in the mouse model and are now only concentrating on the WR strain.

Our currently available vaccinia recombinants (expressing either HtrA or SOD) did not protect against a virulent *Brucella* challenge and at best only induced a humoral immune response specific for the recombinant *Brucella* antigen. Therefore, we have switched to the use of a synthetic early - late promoter to produce vaccinia recombinants to express the genes for the following proteins: GroEL, HtrA, CuZn SOD, YajC, SecD, 18kDa lipoprotein and 14 kDa protein. Mice immunized with these new constructs will be tested for protection against a virulent challenge and their humoral and CMI response will be followed.

We made digoxigenin tagged corresponding oligonucleotides to two previously identified N-terminal sequences of 2 proteins (10.6 kDa and 11.2 kDa) but were unable to demonstrate hybridization to either *E.coli* or *Brucella abortus* chromosomal DNA. Therefore, we have not yet identified the corresponding genes. We prepared antisera to these proteins and are screening libraries to find the corresponding genes. Once identified, genes will be cloned into the vaccinia vector.

We have developed a relatively reliable, multistep method to purify the antigens selected for recombinant production. These antigens are needed for *in vitro* and *in vivo*

immune response characterizations. Alternatively, antigens will be purified using fusion protein production in *E. coli*. A general problem with all these approaches is the limited amount of antigen obtained and LPS contamination. Because of this, we have initiated subcloning into Baculovirus and expect to produce large quantities of the selected antigens through this expression system.

Finally, we are pursuing alternative fractionation methods to identify immunoreactive *Brucella* proteins which play a role in the induction of CMI. The fractionation approach is described and we have tentatively identified 4 new candidate antigens.

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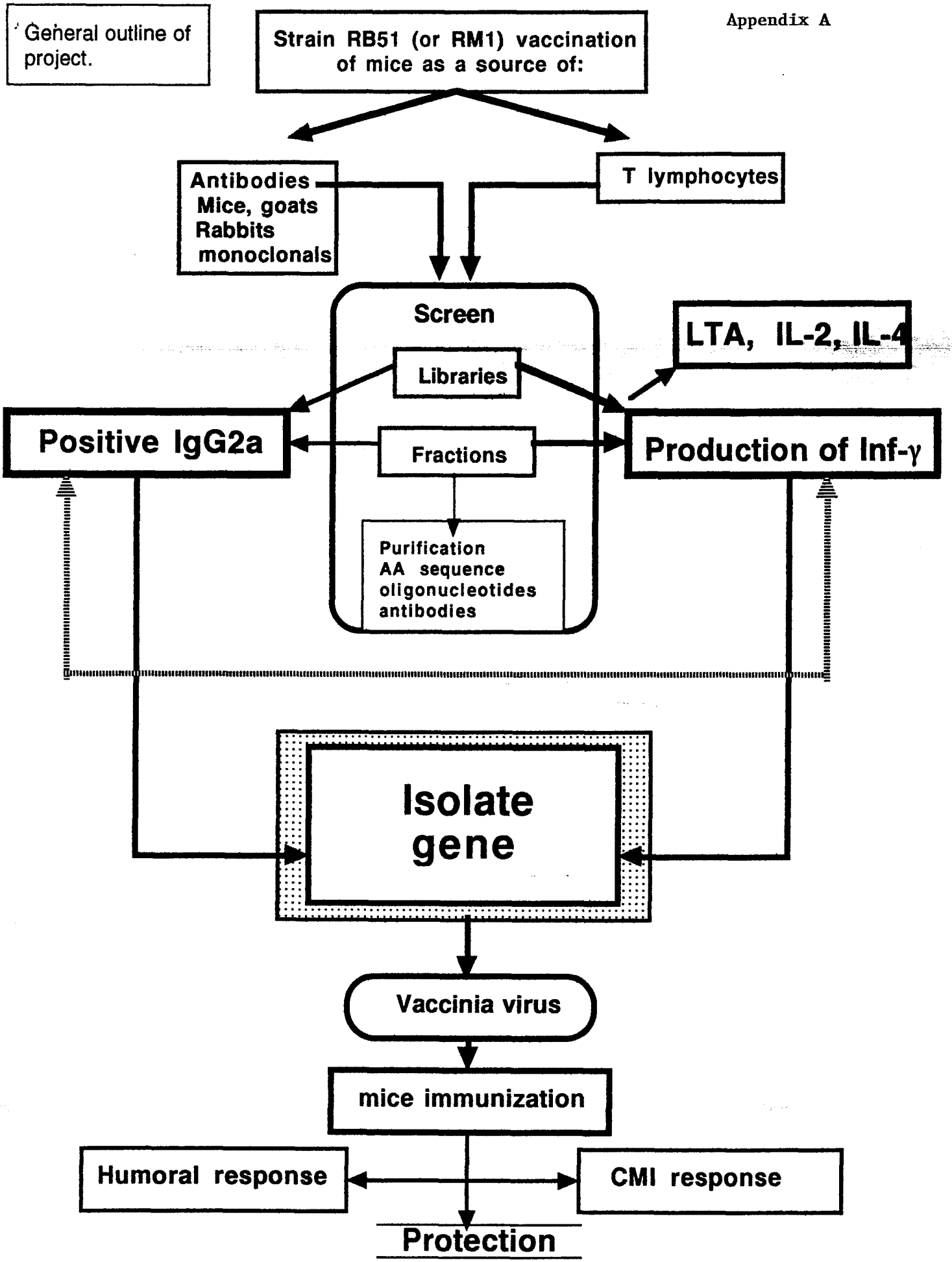
IX. APPENDIX

Appendix A

General outline of project.



ALIGNMENT REPORT

Alignment Report of army grant data, using Clustal method with PAM250 residue weight table.
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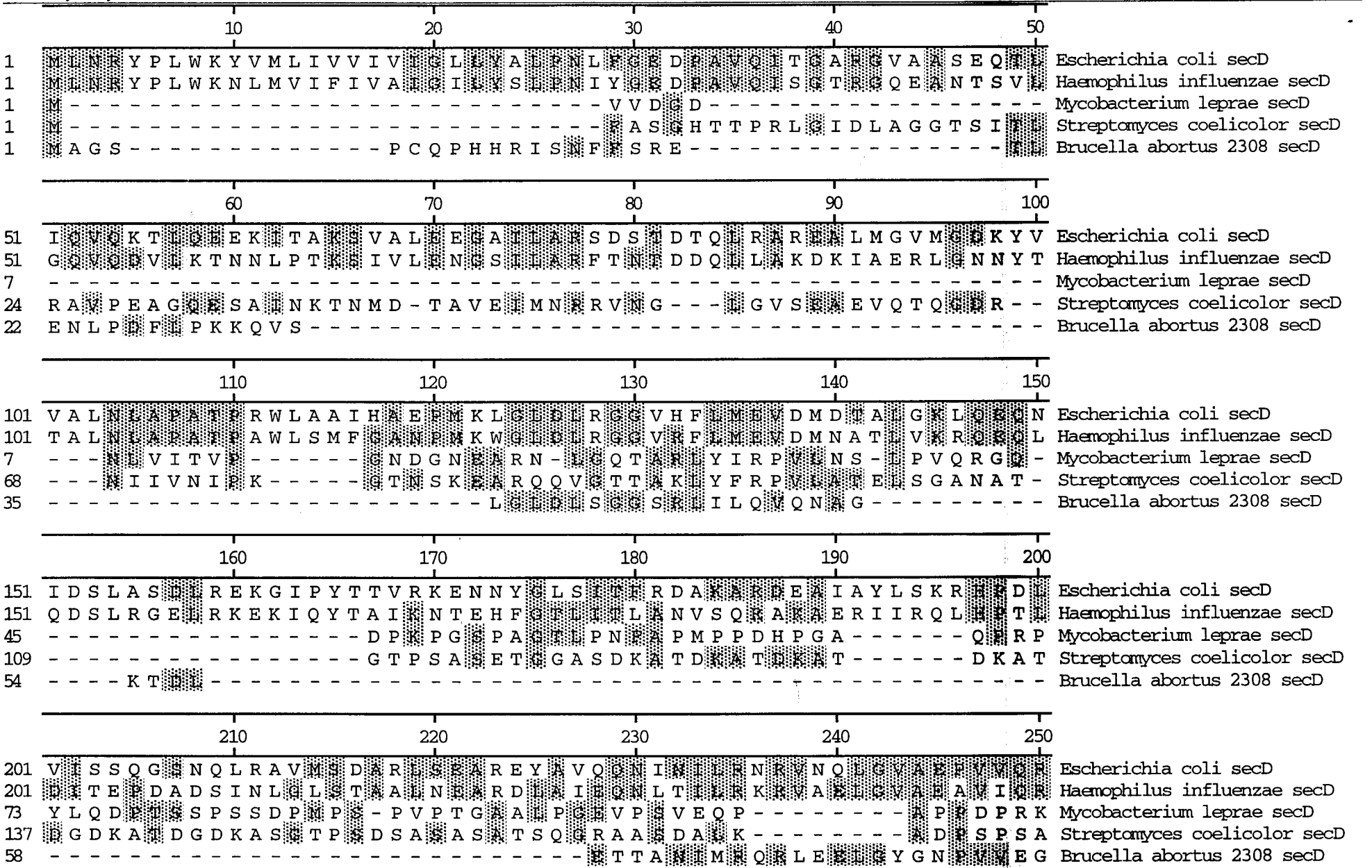
Appendix B



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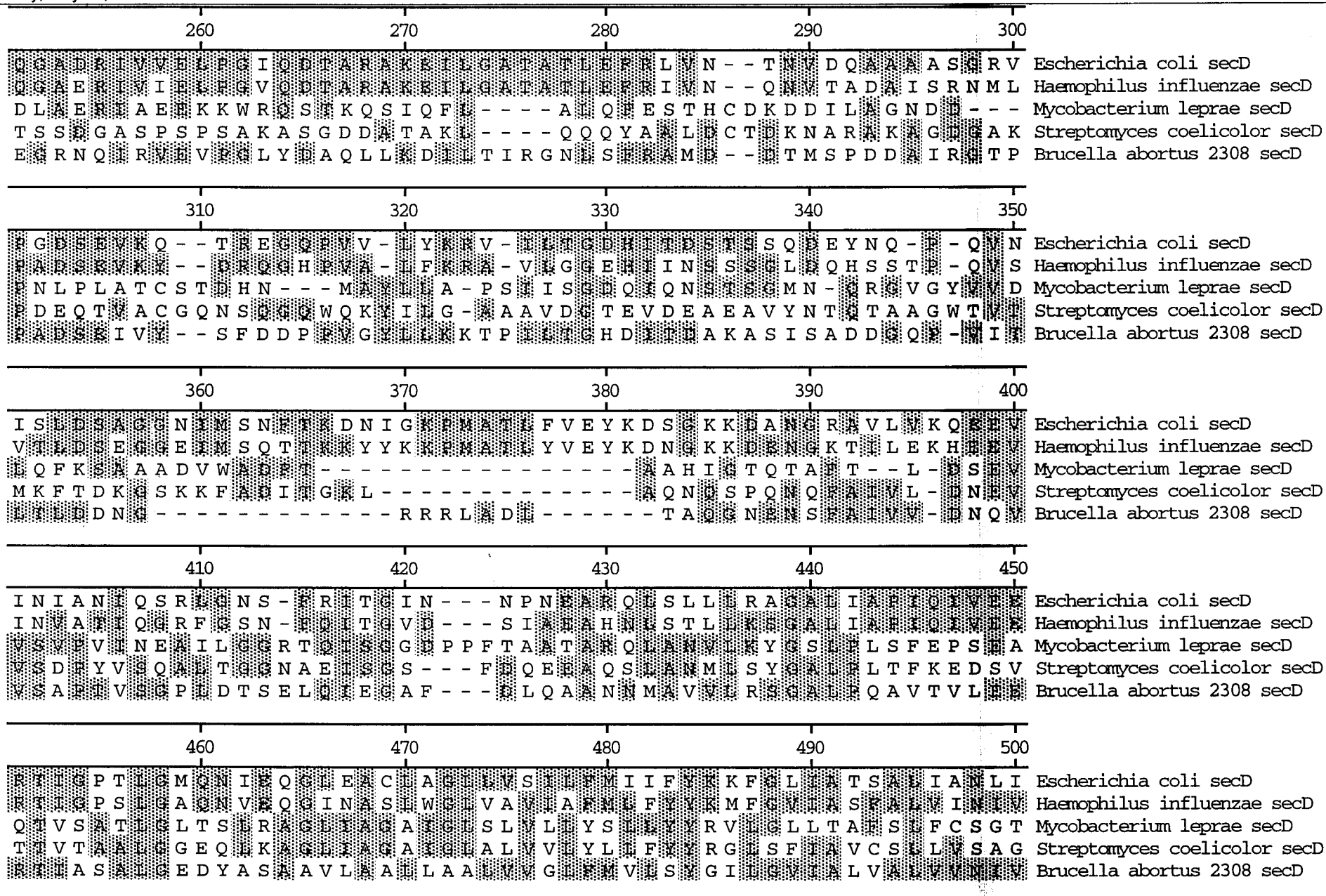

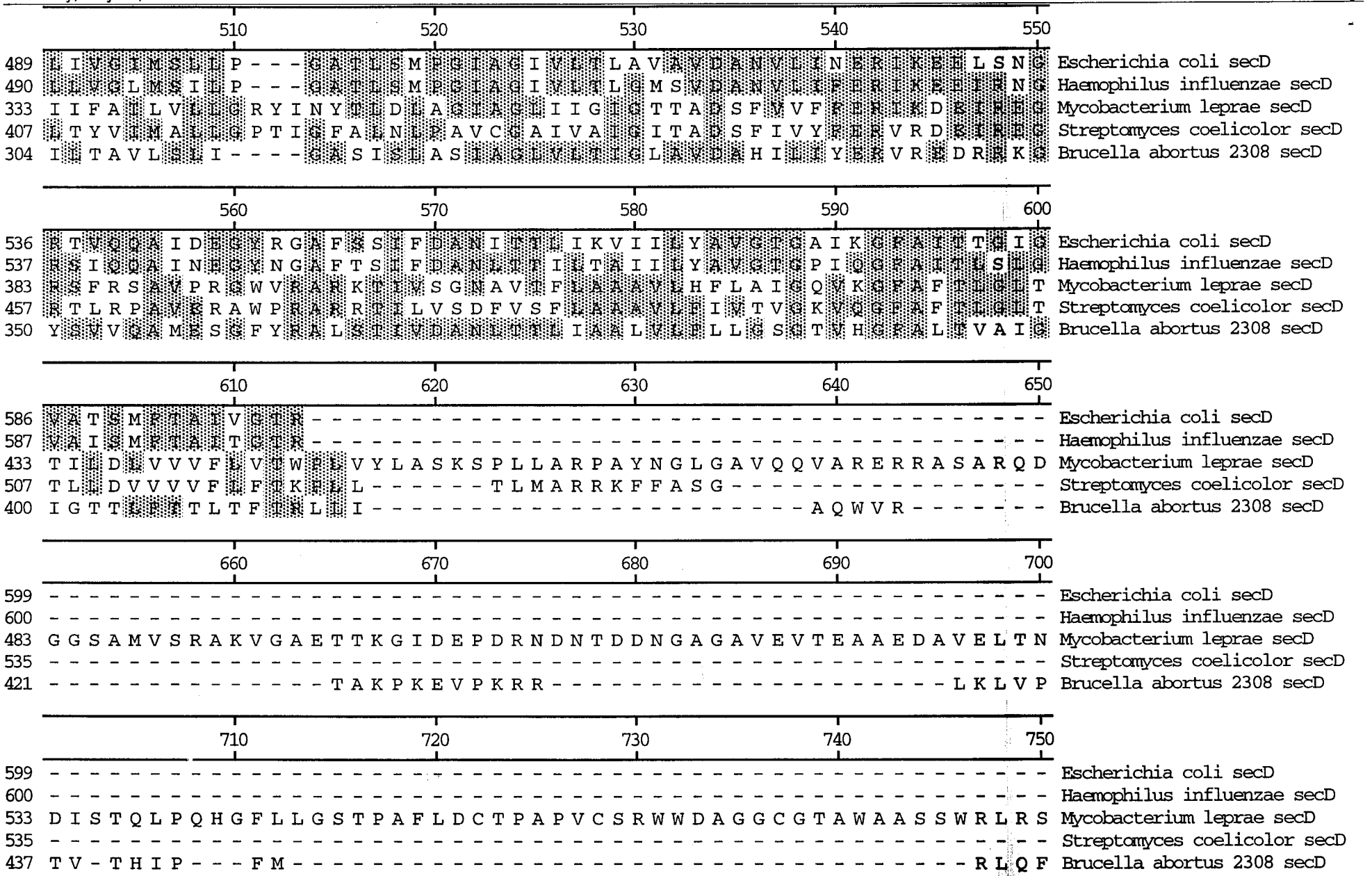
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333 IIFAILVLLGRYINYTLDLAGIAGLIIGIGTTADSFMVFERIKDKIRDG Mycobacterium leprae secD
407 LTYVIMAILGPTIGFALNLPAVCGAIVAIGITADSFIVYERVRDKIAEG Streptomyces coelicolor secD
304 ILTAVLSLI----GASISLASIAGLVLTIGLAVDAHILIYERVREDREKG Brucella abortus 2308 secD
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537 RSIQQAINEGYNGAFTSIFDANLTTIITAIILYAVGTGPIQGFALTLSLG Haemophilus influenzae secD
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587 VAISMFTAITGTR---------- Haemophilus influenzae secD
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535 ---------- Streptomyces coelicolor secD
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Alignment Report of army grant data, using Clustal method with PAM250 residue weight table.
Thursday, May 23, 1996 10:24 AM

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535 -HKWSGL-------------------------------DPKA--LGAKPS Streptomyces coelicolor secD
474 IDFRGGSMVELQARNGDAN----------LEDINERLAELNLD Brucella abortus 2308 secD

                860        870
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616                                               Haemophilus influenzae secD
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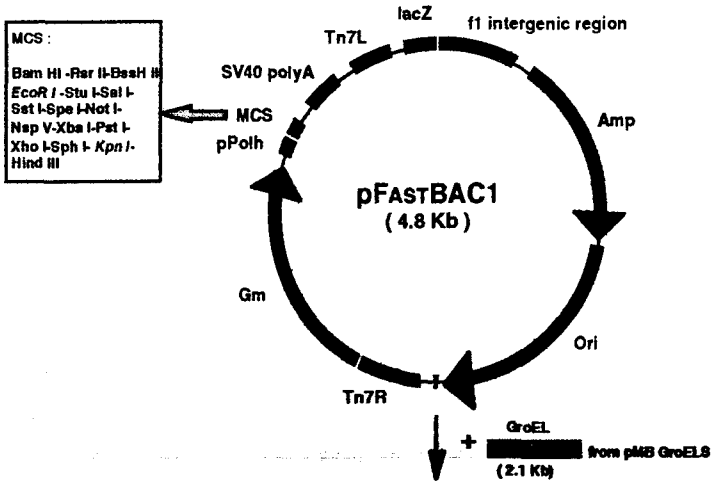
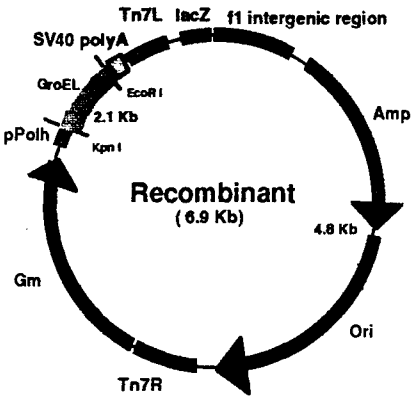
Decoration 'Decoration #1': Shade (with deep red at 40% fill) residues that match the Consensus **exactly**.

Recombinant GroEL





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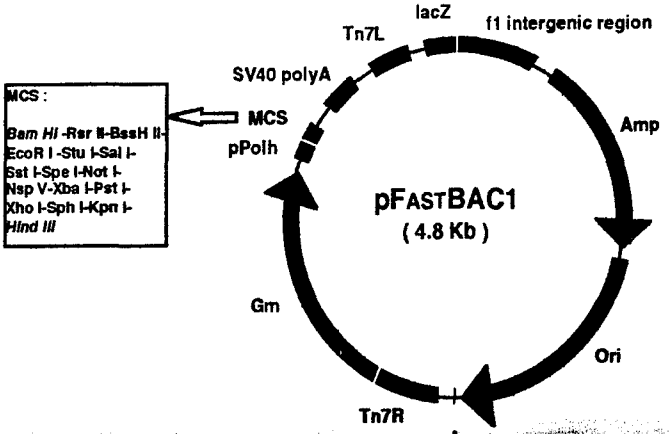
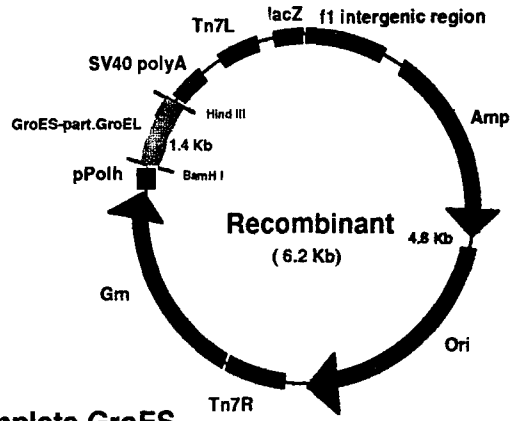
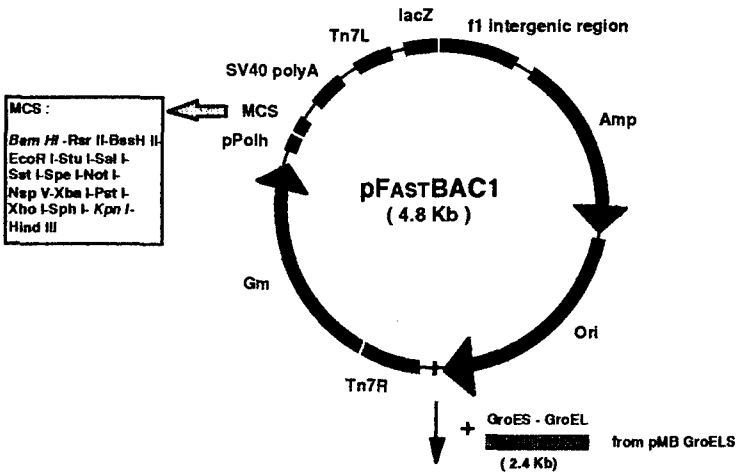
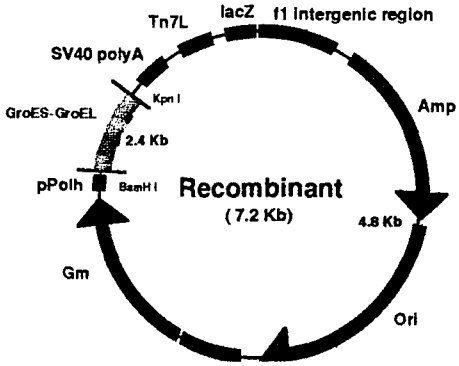




Recombinant Complete GroEL-Complete GroES





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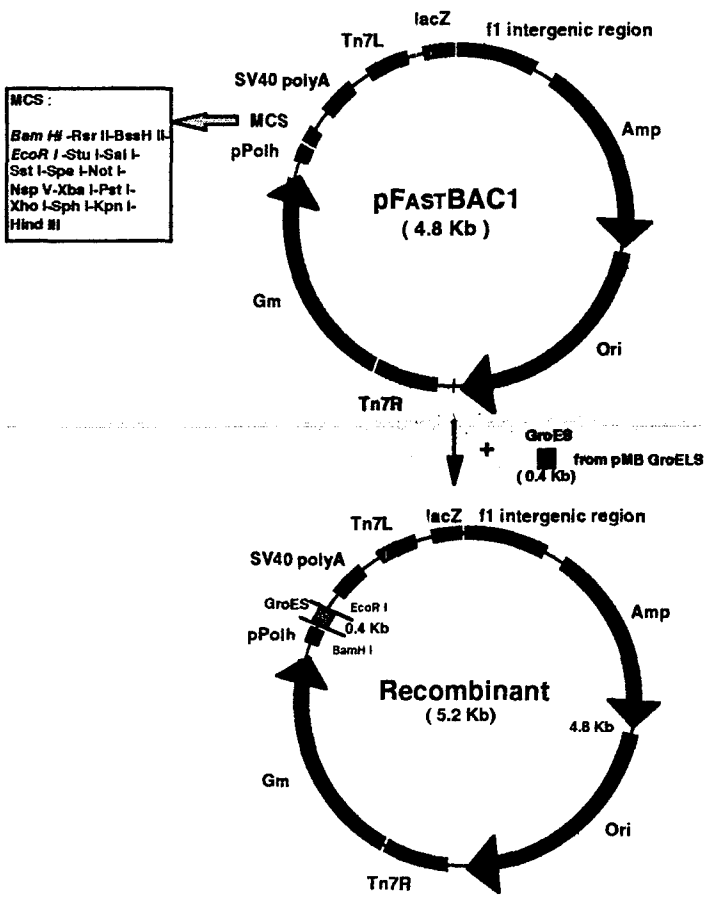

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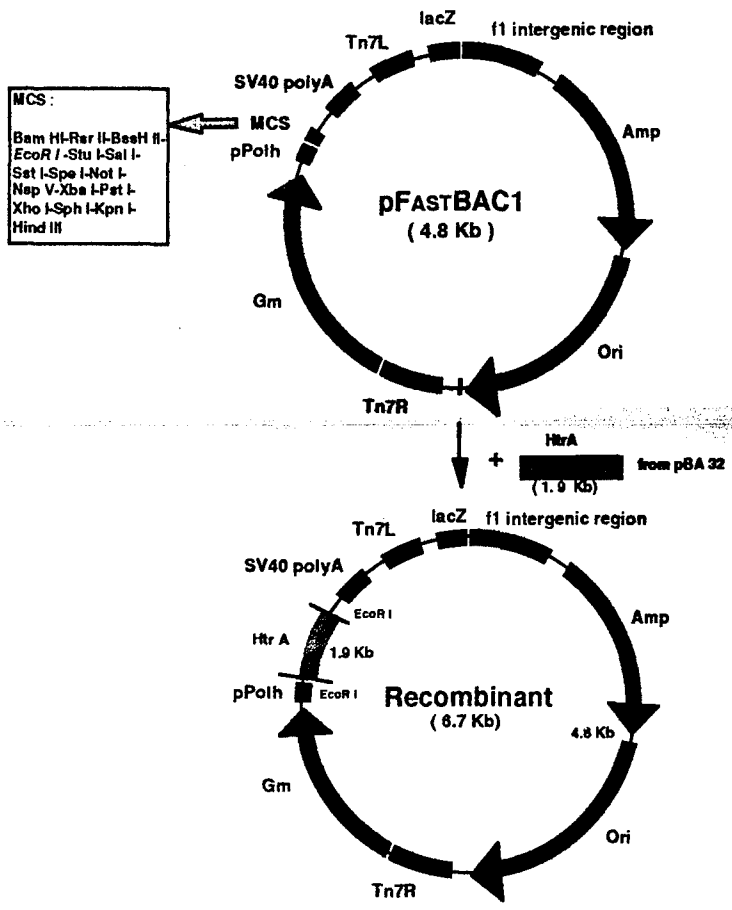

PLASMIDS FOR VACCINIA TRANSFECTIONS